Effect of thioacetamide on cytochrome *P*-450 synthesis in rat liver

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A single injection of thioacetamide to rats results in a striking decrease in liver cytochrome *P*-450. The drug blocks the phenobarbitone-mediated induction of cytochrome *P*-450b. Under these conditions, there is a decrease in the synthesis of cytochrome *P*-450b, which is essentially due to a decrease in the amount of translatable mRNA for cytochrome *P*-450b. This effect of thioacetamide is not due to a general effect on protein synthesis or polyribosome stability. Multiple injections of thioacetamide also decrease the synthesis of cytochrome *P*-450b and the amounts of its translatable mRNA.

Thioacetamide is a slow-acting hepatocarcinogen. Acute and chronic administration lead to liver cirrhosis and tumours (Fitzhugh & Nelson, 1948). Repeated administration of the chemical leads to enlarged nucleoli and elevated nucleolar RNA polymerase activity (Adams & Busch, 1963; Anderson *et al.*, 1977). A single feeding is associated with a brief suppression of RNA formation, followed by a rebound acceleration of anabolic nuclear RNA synthesis (Busch & Smetana, 1970). An enhanced energy-dependent transport of RNA from nucleus to cytoplasm, after thioacetamide treatment, has also been reported (Clawson *et al.*, 1980). Repeated administration of thioacetamide has also been reported to enhance albumin synthesis and the amounts of translatable albumin mRNA (Stolf *et al.*, 1970; Chakrabarty & Schneider, 1978). At the same time, thioacetamide treatment has also been reported to suppress the induction of δ -aminolaevulinic synthase and mixed-function oxidase by various chemical agents, and that of tyrosine aminotransferase by cortisol (Yoshida & Neal, 1978; Barker & Smuckler, 1972). Thioacetamide has also been reported to inhibit protein synthesis when fed at a concentration of 20 mg/100 g body wt. (Barker *et al.*, 1963).

Thus it is not clear whether the consequences of thioacetamide administration are due to a generalized effect on the synthesis and degradation of macromolecules, with a compensatory rebound mechanism accounting for some of the anabolic effects, or to the chemical having specific effects

on the expression of individual genes. In the present study, the effect of a single injection of thioacetamide on the induction of cytochrome *P*-450 synthesis has been examined in relation to its effect on the overall synthetic and degradative machinery of rat liver.

Materials and methods

Materials

Cytochrome *P*-450b (Ryan *et al.*, 1979) was purified from phenobarbitone-injected rats by the procedure described by West *et al.* (1979). Antibodies to cytochrome *P*-450b were raised in rabbits, and the immunoglobulin G was purified by specific binding to the antigen and subsequent dissociation (Bhat & Padmanaban, 1979). [³⁵S]-Methionine (800 Ci/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. In some experiments [³⁵S]methionine (200 Ci/mmol) prepared from carrier-free [³⁵S]sulphate was also used.

Methods

Treatment of animals. Male rats of the Institute strain (100–110 g) were used in these studies. Thioacetamide (5 mg/100 g body wt.) and phenobarbitone (8 mg/100 g body wt.) were administered intraperitoneally. The animals were killed at different intervals of time after a single injection of the chemicals. In some experiments, the animals received four injections, each at an interval of 24 h.

Processing of livers. The livers from two animals were usually pooled and processed. For measurement of microsomal protein and RNA, the livers were homogenized in TKM medium [10 mM-

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid

Tris/HCl (pH 7.4)/50 mM-KCl/5 mM-MgCl₂] containing 0.25 M-sucrose. The homogenate was spun at 800g for 10 min and the supernatant spun at 10000g for 10 min to remove the nuclear and mitochondrial fractions. The post-mitochondrial fraction was spun at 100000g for 1 h to isolate the microsomal fraction. In experiments involving the determination of the components of the drug-metabolizing complex, the livers were homogenized in 1.15% (w/v) KCl and then processed as described above.

Polyribosome-profile analysis. This was done as described by Shafritz (1974), with some modifications. Livers were homogenized in TKM medium containing 0.25 M-sucrose and 200 µg of heparin/ml. The post-mitochondrial supernatant was treated with sodium deoxycholate and Triton X-100 (each 1%, w/v, final concn.). Lysate (7 ml) was layered over 4 ml of 1 M-sucrose in TKM medium and spun at 150000g for 16 h to isolate total polyribosomes. The pellet was suspended in TKM medium and about 5 A₂₆₀ units were layered on a linear sucrose gradient (10–40%, w/v, in TKM) and spun at 280000g for 75 min. The tubes were punctured at the bottom and the flow-through was scanned for absorbance profiles.

Cytochrome P-450 synthesis by isolated microsomal fraction *in vitro*. The procedure was performed as described by Bhat & Padmanaban (1979). The reaction mixture (total volume 0.25 ml) contained: 0.5 mM-ATP, 0.25 mM-GTP; 8 mM-phosphocreatine; amino acid mixture minus methionine, 40 µg/ml; 1 mM-dithiothreitol; 10 mM-Tris/HCl buffer, pH 7.6; 5 mM-MgCl₂; 60 mM-KCl; 1.2 mg of S₁₀₀ protein/ml; 10 µg of creatine kinase; 80 µCi of [³⁵S]methionine (200–800 Ci/mol); and 4 mg of the microsomal suspension protein. The S₁₀₀ fraction was prepared by passing the post-microsomal supernatant through a Sephadex G-25 column. The mixture was incubated at 37°C for 30 min. Portions (5 µl) were transferred to Whatman filter-paper discs and processed to measure radioactivity incorporated into total trichloroacetic acid-precipitable protein. The rest of the mixture was solubilized in cholate-containing buffer, centrifuged to remove debris, and immunoprecipitated by adding a 2-fold excess of anti-(cytochrome P-450b) antibody (Bhat & Padmanaban, 1978). The washed immunoprecipitate was analysed by SDS/polyacrylamide-gel electrophoresis.

Quantification of cytochrome P-450b mRNA. This was performed with a reticulocyte-lysate cell-free system (Pelham & Jackson, 1976), programmed with poly(A)-containing RNA isolated from the post-mitochondrial supernatant or polyribosomes of liver from animals given the different treatments. Polyribosomes were isolated from liver by the Mg²⁺ precipitation method of Palmiter (1974).

RNA was isolated by the guanidinium chloride method, and poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography (Krystosek *et al.*, 1975). The incubation mixture (volume 100 µl) contained: 10 mM-phosphocreatine; 1 mM-ATP; 20 µM-GTP; 1 mM-dithiothreitol; 20 mM-Hepes, pH 7.6; 80 mM-KCl; 1.5 mM-MgCl₂; 300 µM-spermidine; nuclease-treated lysate; 100 µCi of [³⁵S]methionine; and 8 µg of poly(A)-containing RNA. The mixture was incubated at 37°C for 60 min and then processed for the measurement of synthesis of total protein and cytochrome P-450b as described above, except that SDS and Triton X-100 were used for solubilization and *Staphylococcus aureus* cells used for adsorbing the antigen-antibody complex (Maccecchini *et al.*, 1979).

SDS/polyacrylamide-gel electrophoresis. The labelled antigen-antibody complexes were dissociated with 2% (w/v) SDS/50 mM-Tris/HCl (pH 6.8)/5% (w/v) 2-mercaptoethanol in a boiling-water bath for 2 min and analysed on 8%-acrylamide gels as described by Laemmli (1970). The gels were stained with Coomassie Blue R and the band corresponding to cytochrome P-450 was cut out, digested with H₂O₂ and its radioactivity measured. In some experiments, the gels were subjected to fluorography (Laskey & Mills, 1975).

Measurement of total cytochrome P-450, cytochrome P-450b, microsomal haem and δ-aminolaevulinate synthesis activity. Total cytochrome P-450 was measured in microsomal suspensions by the spectral method (Omura & Sato, 1964). Cytochrome P-450b was measured in microsomal preparations, after solubilization, by the radial-immunodiffusion procedure (Thomas *et al.*, 1979). Total microsomal haem content was measured by the alkali-pyridine haemochromogen method (Porra & Jones, 1963). δ-Aminolaevulinate synthase activity was assayed in liver homogenates by measuring δ-aminolaevulinate by the colorimetric procedure, after column fractionation (Marver *et al.*, 1966).

Results

The results presented in Table 1 indicate that a single injection of thioacetamide has no significant effect on liver weight. The microsomal protein and RNA contents show a statistically significant increase at 48 h after thioacetamide administration, and the values tend to revert to normal around 96 h. The results presented in Table 2 indicate that the microsomal cytochrome P-450 contents decrease by 50% at 24 h after the drug administration. Maximal decrease occurs at 48 h, and the contents remain depressed even at 96 h. Cytochrome b₅ contents show a similar response, but the decrease

Table 1. *Effect of a single injection of thioacetamide on liver weight, microsomal protein and RNA contents*

Thioacetamide (5 mg/100 g body wt.) was administered intraperitoneally and the animals were killed at different intervals of time. The values given represent the means $\pm$ S.D. for five animals.

Time of killing after thioacetamide administration (h)	Liver wt. (g)	Microsomal protein (mg/g of liver)	Microsomal RNA (mg/g of liver)
0	5.5 $\pm$ 0.2	16.9 $\pm$ 1.1	1.4 $\pm$ 0.2
24	5.1 $\pm$ 0.3	17.9 $\pm$ 1.5	1.5 $\pm$ 0.1
48	5.4 $\pm$ 0.2	23.3 $\pm$ 0.9	1.9 $\pm$ 0.1
72	5.6 $\pm$ 0.3	19.6 $\pm$ 0.7	1.6 $\pm$ 0.1
96	5.2 $\pm$ 0.2	17.1 $\pm$ 1.0	1.4 $\pm$ 0.2

Table 2. *Effect of a single injection of thioacetamide on the contents of cytochrome P-450 and cytochrome b₅ and on NADPH-cytochrome c reductase activity*

The experimental details are given in text and Table 1.

Time of killing after thioacetamide administration (h)	Cytochrome P-450 (nmol/mg of protein)	Cytochrome b ₅ (nmol/mg of protein)	NADPH-cytochrome c reductase (nmol of cytochrome c reduced/min per mg of protein)
0	0.65 $\pm$ 0.08	0.33 $\pm$ 0.03	25.9 $\pm$ 1.7
24	0.34 $\pm$ 0.02	0.29 $\pm$ 0.02	22.3 $\pm$ 1.8
48	0.14 $\pm$ 0.03	0.14 $\pm$ 0.01	20.1 $\pm$ 2.6
72	0.27 $\pm$ 0.03	0.25 $\pm$ 0.03	19.9 $\pm$ 2.9
96	0.32 $\pm$ 0.04	0.30 $\pm$ 0.04	26.2 $\pm$ 1.3

Table 3. *Effect of a single injection of thioacetamide on the induction of cytochrome P-450, δ -aminolaevulinate synthase and microsomal haem in rat liver*

The animals were given the drug treatments for a period of 24 h. The experimental conditions are described in the text. The values are averages from two experiments, in which two livers were pooled in each experiment. Abbreviation: ND, not detectable.

Treatment	Cytochrome P-450 (nmol/mg of protein)	δ -Aminolaevulinate synthase (nmol of δ -aminolaevulinate/ g of liver)	Microsomal haem (nmol/mg of protein)
Control	0.61	15.2	0.98
Phenobarbitone	1.31	32.4	2.12
Thioacetamide	0.31	ND	0.77
Phenobarbitone + thioacetamide	0.69	18.1	0.76

is not as pronounced as observed for cytochrome P-450. NADPH-cytochrome c reductase activity is affected to an even lesser extent.

To examine the effect of thioacetamide on the induction of cytochrome P-450, and also to follow the effect of the chemical on the synthesis of a specific species of cytochrome P-450, the amounts of total cytochrome P-450 were measured at 24 h after the administration of phenobarbitone and thioacetamide. Table 3 shows that thioacetamide strikingly blocks the phenobarbitone-mediated increase in cytochrome P-450 content. The results also indicate that thioacetamide blocks the phenobarbitone-mediated increase in δ -aminolaevulinate synthase activity, the rate-limiting enzyme of the haem-biosynthetic pathway, and total micro-

somal haem content. Thioacetamide is thus an inhibitor of haem synthesis and decreases the haem pool.

To examine whether the decrease in cytochrome P-450 content measured spectrally is merely due to an inhibition of the synthesis of the prosthetic group required to generate the cytochrome P-450 spectrum, or is due to a decrease in the cytochrome P-450 protein content, the phenobarbitone-inducible cytochrome P-450b species was quantified by radial immunodiffusion under these conditions. The results presented in Fig. 1 and Table 4 indicate that the phenobarbitone-inducible cytochrome P-450b species, which accounts for about 5–8% of the total cytochrome P-450, constitutes nearly 60% of the total at 24 h after the drug

administration. Thioacetamide has a striking inhibitory effect on the phenobarbitone-mediated increase in the cytochrome *P*-450b protein content.

The cytochrome *P*-450b protein content was also estimated by staining with Coomassie Blue R, after separation of the microsomal proteins on SDS/polyacrylamide gels. Fig. 2 indicates that the band corresponding to authentic cytochrome *P*-450b is very much enriched in the microsomal

Fig. 1. Estimation of cytochrome *P*-450b content by radial immunodiffusion

Purified cytochrome *P*-450b or solubilized microsomal fraction were loaded into wells made in the agarose matrix containing 0.1% of anti-(cytochrome *P*-450b) antibody. The rings were allowed to develop over a period of 48 h and then stained with Coomassie Blue R. Row A (left to right): 0.2, 0.4 and 0.6 µg of cytochrome *P*-450b. Row B (left to right): microsomal preparation after phenobarbitone + thioacetamide treatment, 6 h and 24 h (100 µg of protein). Row C: microsomal preparation from control animals (100 µg of protein). Row D (left to right): microsomal preparation from phenobarbitone-treated animals, 6 h and 24 h (30 µg of protein). The protein concentrations used were in the range that produced a linear response for a particular treatment.

preparation from phenobarbitone-treated animals. The microsomal preparation from animals treated with phenobarbitone + thioacetamide shows a significantly decreased concentration of the cytochrome *P*-450b band. The results thus indicate that thioacetamide administration blocks the phenobarbitone-mediated increase in haem as well as the apoprotein content of cytochrome *P*-450.

The effect of thioacetamide administration on cytochrome *P*-450b synthesis was then examined in cell-free systems. The results presented in Fig. 3 and Table 5 indicate that the synthesis of cytochrome *P*-450b by microsomal fractions from the drug-treated rat livers is drastically decreased under conditions of thioacetamide treatment. It is now well established that phenobarbitone treatment leads to a striking increase in cytochrome *P*-450b synthesis and cytochrome *P*-450b mRNA (Bhat & Padmanaban, 1979; Kumar & Padmanaban, 1980; Gonzalez & Kasper, 1982). To examine whether thioacetamide treatment affects the amounts of cytochrome *P*-450b mRNA, poly(A)-containing RNA isolated from drug-treated livers was translated in the reticulocyte cell-free system and the cytochrome *P*-450b synthesized was quantified. The results presented in Fig. 4 and Table 5 clearly reveal that thioacetamide blocks the phenobarbitone-mediated increase in translatable mRNA for cytochrome *P*-450b. The effect of thioacetamide is manifest as early as 6 h after the drug administration. It should be noted that the total protein synthesized in either the homologous or the heterologous cell-free systems is not affected by thioacetamide treatment.

In view of the earlier suggestion that the nucleolar changes brought about by thioacetamide are perhaps due to the activation and release of latent lysosomal ribonuclease (Busch & Smetena, 1970), the possible influence of such an effect on the polyribosome profiles was determined at different times after the drug treatments. A 30% decrease in the recovery of total polyribosomes was

Table 4. Effect of a single injection of thioacetamide on the phenobarbitone-mediated increases in total cytochrome *P*-450 and cytochrome *P*-450b contents in rat liver microsomal fractions

Total cytochrome *P*-450 was measured spectrally, and cytochrome *P*-450b by the radial-immunodiffusion method. Other experimental details are given in the text.

Time of killing after a single administration of drug(s) (h)	Total cytochrome <i>P</i> -450 (nmol/mg of protein)		Cytochrome <i>P</i> -450b (nmol/mg of protein)	
	Phenobarbitone	Phenobarbitone + thioacetamide	Phenobarbitone	Phenobarbitone + thioacetamide
0	0.68	0.65	0.04	0.05
12	1.42	0.74	0.84	0.07
24	1.63	0.76	1.03	0.09
48	1.75	0.86	1.13	0.10
72	1.51	0.62	0.94	0.08
96	0.91	0.59	0.52	0.06

Fig. 2. SDS/polyacrylamide-gel electrophoresis of microsomal preparations

Solubilized microsomal preparations (100 μ g of protein) were analysed on SDS/polyacrylamide gels (8%) and stained with Coomassie Blue R. The treatments given to the rats for 24 h were as follows: slot 1, phenobarbitone + thioacetamide; slot 2, phenobarbitone; slot 3, control. Arrow indicates the position of authentic cytochrome *P*-450b.

noticed at 15 h after phenobarbitone + thioacetamide treatment. No significant change was observed at 6, 24 or 48 h, and even at 15 h the polyribosome profiles for phenobarbitone- and phenobarbitone + thioacetamide-treated livers were quite similar (results not shown).

Finally, the effect of multiple injections of thioacetamide on cytochrome *P*-450 content and synthesis was examined. Four injections of the drug were found to depress the cytochrome *P*-450 content. Under these conditions, the drug was found to inhibit strikingly the phenobarbitone-mediated increase in cytochrome *P*-450b synthesis as well as the cytochrome *P*-450b mRNA, as revealed by translation studies *in vitro*. Total protein synthesis was depressed by about 15–20% under these conditions (results not shown).

Discussion

The results of the present investigation clearly reveal that a single injection of thioacetamide does not give any gross liver toxicity at the biochemical level. A slight anabolic effect is seen at 48 h, when there is an increase in microsomal protein and RNA contents. However, thioacetamide depresses the contents of cytochrome *P*-450 strikingly, and

Fig. 3. Effect of thioacetamide on phenobarbitone-mediated induction of cytochrome *P*-450b synthesis in homologous cell-free systems

Protein synthesis was performed with microsomal fractions isolated from animals given the different treatments for a period of 24 h. Experimental details are given in the text. At the end of the incubation, the microsomal preparations were solubilized, immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. The amount of protein radioactivity (trichloroacetic acid precipitable) taken for immunoprecipitation in each case was 1.3×10^6 c.p.m. The treatments were as follows: slot 1, phenobarbitone + thioacetamide; slot 2, control; slot 3, phenobarbitone; slot 4, thioacetamide. Arrow indicates the position of cytochrome *P*-450b.

the effect persists even 96 h after a single injection of the drug.

Thioacetamide is also able to block the induction of cytochrome *P*-450b by phenobarbitone. There is an inhibition of the actual synthesis of the cytochrome *P*-450b protein species, which in turn is due to a decrease in the mRNA for cytochrome *P*-450b. The decreased translatable activity of cytochrome *P*-450b mRNA can be correlated to its decreased amount, although changes in translatable activity owing to alteration in secondary structure cannot be entirely ruled out. Quantification by the hybridization procedure by using a cloned probe would be necessary to confirm this

Table 5. Effect of thioacetamide administration on phenobarbitone-mediated increase in cytochrome P-450b synthesis in homologous and heterologous cell-free systems *in vitro*

For the homologous translation, microsomal fractions were isolated from treated animals and protein synthesis was performed in the presence of the S_{100} fraction prepared from normal animals. For heterologous translation, poly(A)-containing RNA was isolated from the treated-rat liver polyribosomes and equal amounts were translated in the rabbit reticulocyte-lysate system. In both experiments, the animals were treated with the chemicals for a period of 6h. Cytochrome P-450b synthesis was quantified by using the specific antibody and SDS/polyacrylamide-gel analysis of the immunobound material. Other experimental details are given in text.

Treatment	Homologous translation		Heterologous translation	
	$10^{-6} \times$ Total protein synthesized (c.p.m./mg of protein)	Cytochrome P-450b synthesized (% of total)	$10^{-5} \times$ Total protein synthesized [c.p.m./ μ g of poly(A)-containing RNA]	Cytochrome P-450 synthesized (% of total)
Control	1.2	0.22	3.1	0.12
Phenobarbitone	1.4	3.13	3.5	2.20
Phenobarbitone + thioacetamide	1.01	0.29	3.2	0.14
Thioacetamide	1.1	0.14	3.5	0.08

Fig. 4. Effect of thioacetamide on phenobarbitone-mediated increase in translatable cytochrome P-450b mRNA. Poly(A)-containing RNA was isolated from the polyribosomes of animals given the different treatments for a period of 6h. Poly(A)-containing RNA (8μ g in each case) was translated in the reticulocyte lysate system (100μ l) and then processed for immunobinding, SDS/polyacrylamide-gel analysis and fluorography. The amount of protein radioactivity (trichloroacetic acid-precipitable) taken in each case was 3×10^5 c.p.m. The treatments were as follows: slot 1, control; slot 2, phenobarbitone; slot 3; thioacetamide; slot 4, phenobarbitone + thioacetamide. Arrow indicates the position of authentic cytochrome P-450b.

point. The effect of thioacetamide in depressing cytochrome P-450 synthesis appears to be a specific effect in the sense that the total protein-synthesis rates are not affected. However, with thioacetamide + phenobarbitone treatment there is a transient decrease of 30% in total protein synthesis and polyribosome recovery. This effect is not seen at 6 or 24h after the drug treatments. The reason for this transient effect of thioacetamide + phenobarbitone at 15h after treatment is not clear. The inhibitory effect of thioacetamide on cytochrome P-450 content and synthesis can be seen as early as 6h or as late as 72h after the drug administration.

The decrease in cytochrome P-450b mRNA under conditions of thioacetamide treatment may indicate a block in cytochrome P-450 gene transcription, although other factors, such as stability of mRNA, processing and translocation of the precursor RNA across the nuclear membrane, should also be considered. However, translation studies with poly(A)-containing RNA isolated from the post-mitochondrial supernatant or polyribosomes give similar results in terms of the effect of thioacetamide administration on translatable cytochrome P-450b mRNA. This indicates that the drug effect is not at the level of conversion of inactive mRNA bound to ribonucleoprotein particles in the cytoplasm into active mRNA bound to polyribosomes.

Since thioacetamide inhibits haem synthesis, the question arises whether the prosthetic group regulates cytochrome P-450 gene transcription and/or other post-transcriptional steps. However, administration of exogenous haemin does not counteract the effects of thioacetamide in any of the parameters investigated in the present study

(results not shown). But on this result alone it becomes difficult to eliminate the possible involvement of haem in cytochrome *P*-450 gene expression, since classical inhibitors of haem synthesis such as cobalt and 3-amino-1,2,4-triazole have also been found to block the phenobarbitone-mediated increase in cytochrome *P*-450b mRNA (Ravishankar & Padmanaban, 1983). Further studies are needed to understand the possible involvement of haem in cytochrome *P*-450 gene expression. It would also be of interest to examine whether thioacetamide acts through a common mechanism to block the induction of cytochrome *P*-450 and cytochrome *b*₅, as well as other proteins such as δ -aminolaevulinate synthase and tyrosine aminotransferase, although it is clear that the drug is not an inhibitor of general protein synthesis under the experimental protocol of the present study.

The effect of multiple injections of thioacetamide in decreasing cytochrome *P*-450 mRNA observed in the present study contrasts with the increase in albumin mRNA observed under similar conditions (Chakrabarty & Schneider, 1978). It would be of interest to examine whether this reciprocal relationship is casual or there is a special significance to the differential expression of the two genes. Finally, the striking and prolonged inhibitory effect of thioacetamide on cytochrome *P*-450 contents and the associated drug-metabolizing activity may be responsible for its action as a slow-acting hepatocarcinogen, since the formation of its active metabolite, thioacetamide *S*-oxide (Yoshida & Neal, 1978), involves the cytochrome *P*-450-dependent mixed-function oxidase system.

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